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Review

Crosstalk between the TGF-β and WNT signalling pathways during cardiac fibrogenesis*

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Cardiac fibrosis is referred to as an excessive accumulation of stromal cells and extracellular matrix proteins in the myocardium. Progressive fibrosis causes stiffening of the cardiac tissue and affects conduction of electrical impulses, leading to heart failures in a broad range of cardiac conditions. At the cellular level, activation of the cardiac stromal cells and myofibroblast formation are considered as hallmarks of fibrogenesis. At the molecular level, transforming growth factor β (TGF-β) is traditionally considered as a master regulator of the profibrotic processes. More recently, the WNT signalling pathway has also been found to be implicated in the development of myocardial fibrosis. In this review, we summarize current knowledge on the involvement of TGF-β and WNT downstream molecular pathways to cardiac fibrogenesis and describe a crosstalk between these two profibrotic pathways. TGF-β and WNT ligands bind to different receptors and trigger various outputs. However, a growing body of evidence points to cross-regulation between these two pathways. It has been recognized that in cardiac pathologies TGF-β activates WNT/β-catenin signalling, which in turn stabilizes the TGF-β/Smad response. Furthermore both, the non-canonical TGF-β and non-canonical WNT signalling pathways, activate the same mitogen-activated protein kinases (MAPKs): the extracellular signal-regulated kinase (Erk), the c-Jun N-terminal kinases (JNKs) and p38. The crosstalk between TGF-β and WNT pathways seems to play an essential role in switching on the genetic machinery initiating profibrotic changes in the heart. Better understanding of these mechanisms will open new opportunities for development of targeted therapeutic approaches against cardiac fibrosis in the future.

Key words: TGF-beta, Smad, WNT, beta-catenin, RhoA-ROCK, p38, JNK, Erk1/2, MAPK, cardiac fibrosis, tissue remodelling, cardiac fibroblasts, heart

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Abbreviations: α-SMA, alpha-smooth muscle actin; ATF3, activating transcription factor 3; ECM, extracellular matrix proteins; Erk, extracellular signal-regulated kinas; GSK-3β, glycogen synthase kinase-3β; IL, interleukin; JNK, c-Jun N-terminal kinase; LEF, lymphoid enhancer factor; LOX, lysyl oxidase; LRP, lipoprotein receptor-related protein; MAPKs, mitogen-activated protein kinases; MAPKK, MAPK kinase; MAPKKK, MAPKK kinase; MRTF, myocardin-related transcription factors; mTOR, mammalian target of rapamycin; PAI-1, plasminogen activator inhibitor-1; PI3K, phosphatidylinositol-3-kinase; PKC, protein kinase C; PLC, phospholipase C; ROCK, Rho-associated, coiled-coil-containing protein kinase; sFRPs, secreted Frizzled-related proteins; Smad, homologues of the *Caenorhabditis elegans* "Sma" and *Drosophila* "MAD" ("Mothers Against Decapentaplegic"); SRF, serum response factor; TAK1, TGF-β-activated kinase 1; TAZ, transcriptional coactivator with PDZ-binding motif; TCF, T-cell factor; TGF-β, transforming growth factor β; TIMP-1, tissue inhibitor of metalloproteinase 1; TRPC6, transient receptor potential canonical 6; uPA, urokinase-type plasminogen activator; WIF1, WNT-inhibitory factor 1; WISP-1, WNT1-induced secreted protein-1; WNT, wingless-type MMTV integration site family member; YAP, yes-associated protein 1

INTRODUCTION

Cardiac fibrosis is characterized as an excessive accumulation of stromal cells and extracellular matrix proteins (ECM) in the myocardium (Li *et al*., 2018). A broad range of pathologic cardiac conditions including myocardial infarction, hypertension, myocarditis, hypertrophic or dilated cardiomyopathy is associated with cardiac tissue remodelling and fibrosis development (Kong *et al*., 2014). Fibrogenesis in the heart can be considered either as a repair or pathogenic process. In case of substantial loss of native cardiac tissue, as for example following myocardial infarction, the damaged tissue is replaced by stromal cells preventing organ rupture. In cardiac conditions without a loss of healthy tissue, fibrogenesis has no evident benefits and should be classified as a pathogenic process. Fibrosis causes not only stiffening of the cardiac tissue leading to impaired mechanical contraction, but also can affect conduction of electrical impulses. Progressive fibrosis is, therefore, one of the major causes of heart failure.

Fibroblasts and myofibroblasts represent the most extensively characterised stromal cell types involved in fibrotic processes in the heart. In the traditional view, resident cardiac fibroblasts become activated, proliferate and became myofibroblasts, which produce an excessive amount of ECM proteins, such as collagen type I and III. Myofibroblasts are characterised by expression of alpha-smooth muscle actin (α-SMA), which upregulates their contractile activity and represents the most reliable marker of these cells (Gabbiani, 2003; Kurose & Mangmool, 2016; Travers *et al*., 2016). Growing body of evidence indicated that other cells present in the heart, including bone marrow-derived cells, epicardial epithelial cells and endothelial cells, can also contribute to myofibroblasts' formation (Haudek *et al*., 2006; Kania *et al*., 2009; Zeisberg *et al*., 2007; Zhou & Pu, 2011). Activation of fibroblasts and myofibroblast lineage differentiation is induced by various environmental stimuli and intracellular molecules including profibrotic factors, such as transforming growth factor β (TGF-β), the WNT proteins, members of renin-angiotensin-aldosterone system, yes-associated protein 1 (YAP)/transcriptional coactivator with PDZ-binding motif (TAZ) transcriptional regu-

lators or various cytokines and chemokines (Kong *et al*., 2014; Piersma *et al*., 2015). These multiple profibrotic inputs activate a complex signalling network that orchestrates fibrotic response at the cellular and organ levels. To build a comprehensive model of cardiac fibrogenesis, a better understanding of crosstalks between individual pathways is needed. In this review, we specifically discuss the TGF-β and WNT downstream molecular pathways activated during cardiac fibrogenesis, with a special focus on the interplay between them.

TGF-β-DEPENDENT SIGNALLING PATHWAYS IN CARDIAC FIBROSIS

TGF-β is a ubiquitously expressed pleiotropic cytokine controlling numerous cellular processes, including proliferation, differentiation, cytoskeletal reorganisation, and ECM protein synthesis. Beside its role in the development, cancer and in maintenance of immunological tolerance, TGF-β is considered as a master regulator of fibrogenesis (Meng *et al*., 2016; Yoshimura & Muto, 2011). TGF-β protein occurs in three isoforms: TGF-β1, -β2 and -β3, which are encoded by distinct genes, but bind to the same receptors (Yoshimura & Muto, 2011). After translation, the TGF-β protein is produced in a latent form. Activation of TGF-β requires proteolytic cleavage and separation of two polypeptides from the active TGF-β form. Plasma membrane-bound integrins and extracellular proteases, such as plasmin and certain matrix metalloproteinases, are typically involved in cleavage of the latent TGF-β complex (Tran, 2012). Active TGF-β binds to the transmembrane TGF-β type II receptor, which in turn recruits and activates the TGF-β type I receptor. This stimulation initiates signal transduction through a canonical Smad-dependent and a number of Smad-independent signalling pathways. The ultimate consequence of the TGF-β signalling is transcriptional dysregulation of target genes which control cell proliferation and production of structural and ECM proteins, such as collagens, laminins, fibronectin and many others (Branton & Kopp, 1999). TGF-β-dependent mechanisms contribute to fibrotic processes not only in the heart, but also in other organs, including kidney, liver, lung or skeletal muscle (Biernacka *et al*., 2011; Dooley & ten Dijke, 2012; Germano *et al*., 2009; Leask, 2007; Meng *et al*., 2015; Paw *et al*., 2017; Tatler & Jenkins, 2012, Li *et al*., 2004).

TGF-β represents a key profibrotic cytokine in cardiac fibrogenesis and inhibition of its action successfully reduced or prevented fibrosis development in various animal models of cardiac disorders. In myocardial infarction model, TGF-β blockage with anti-TGF-β antibodies reduced collagen deposition and affected ECM protein production. Dysregulated tissue remodelling and insufficient scar formation in post-infarcted heart resulted in an increased mortality due to tissue rupture (Frantz *et al*., 2008; Ikeuchi *et al*., 2004). In a rat model of pressure overload, neutralizing anti-TGF-β antibody successfully suppressed fibrosis and thereby prevented the development of diastolic dysfunction (Kuwahara *et al*., 2002). Similar anti-fibrotic effects of anti-TGF-β antibody delivery were observed in a mouse model of experimental autoimmune myocarditis (Kania *et al*., 2009) and in a genetic model of spontaneous hypertrophic cardiomyopathy (Teekakirikul *et al*., 2010). In a pressure overload model, the delivery of anti-TGF-β antibody inhibited profibrotic changes in cardiac interstitial cells, but not in cardiomyocytes. Interestingly, cardiomyocyte-specific deletion of TGF-β type II receptor suppressed not only cardiac hypertrophy, but also tissue remodelling induced by aortic banding, suggesting interplay between different cellular subsets and paracrine action of various profibrotic factors during cardiac fibrogenesis (Koitabashi *et al*., 2011).

Integrins and plasmin play important roles in proteolytic activation of TGF-β. In cardiac fibroblasts, αvβ5 and αvβ3 integrins promote latent TGF-β1 activation and control myofibroblast differentiation (Sarrazy *et al*., 2014). It has been shown that activity of urokinase-type plasminogen activator (uPA) – an enzyme converting plasminogen to plasmin, also controls tissue remodelling in the heart. Accordingly, mice overexpressing uPA developed spontaneous cardiac fibrosis (Moriwaki *et al*., 2004), whereas mice lacking this enzyme showed impaired scar formation in the post-infarcted heart (Heymans *et al*., 1999). Plasminogen activator inhibitor-1 (PAI-1) is a potent inhibitor of uPA. It has been reported that lack of PAI-1 enhanced myocardial fibrosis in aged mice. Importantly, PAI-deficient mice showed elevated TGF-β1 and 2 levels, as well as activation of the TGF-β downstream signalling pathway (Ghosh *et al*., 2010). All of these data highlighted the pivotal role of TGF-β in cardiac fibrosis.

Smad-dependent signalling

The canonical response to TGF-β involves Smad proteins and is referred to as Smad-dependent. Based on their function, Smad proteins can be classified into three categories: receptor activated Smads (R-Smad), common mediator Smads (Co-Smad) and inhibitory Smads (I-Smad). The TGF-β type I receptor specifically recognizes R-Smads (Smad2 and Smad3) and phosphorylates them. Phosphorylated R-Smads bind to the Co-Smad protein (Smad4) forming a functional Smad2/3/4 complex, which is translocated into nucleus and takes part in binding to the DNA sequences. Selectivity and affinity of the Smad2/3/4 complex for specific DNA structures is low and activation of a specific promoter strongly depends on interaction of the Smad2/3/4 complex with other transcription factors. In contrast to R-Smads and Co-Smad, I-Smads (Smad6 and Smad7) are involved in repression of signal transduction. Smad6 competes with Smad4 for binding to R-Smad, whereas Smad7 binds to the TGF-β receptors (Hata *et al*., 1998; Hayashi *et al*., 1997; Mehra & Wrana, 2002). Smad-dependent pathway is the main transducer of the TGF-β signalling and has been shown to play a key role in cardiac fibrogenesis in a number of *in vivo* and *in vitro* models. In a rat model of myocardial infarction, increased TGF-β levels in the scar tissue correlated with elevated abundance of Smad2, Smad3 and Smad4 proteins (Hao *et al*., 1999). Furthermore, Wang *et al*. demonstrated that an elevated level of TGF-β parallels with reduced amount of inhibitory Smad7 in the infarcted hearts for up to 8 weeks (Wang *et al*., 2002). The functional contribution of the Smad-dependent pathway following myocardial infarction has been confirmed in Smad3-deficient mice, which showed reduced collagen III production and attenuated fibrosis in the infarcted hearts (Bujak *et al*., 2007). Smad3-deficient mice also developed reduced fibrosis induced by angiotensin II infusion (Huang *et al*., 2010). In the pressure overload model, TGF-β-Smad signalling in cardiac fibroblasts has been also implicated in the development of myocardial fibrosis. Fibroblast-specific deletion of *Tgfbr1/2* or *Smad3*, but not *Smad2*, markedly reduced the pressure overload-induced fibrosis and inhibited

synthesis of the ECM proteins (Khalil *et al*., 2017). Furthermore, *ex vivo* study on rat cardiac fibroblasts demonstrated that TGF-β-induced collagen synthesis requires the Smad3/4 activity (Shyu *et al*., 2010). Mechanistically, pharmacological inhibition of Smad3/4 in the TGF-β-activated fibroblasts suppressed transcription of endoglin – a membrane glycoprotein constituting a part of the TGF-β receptor complex. In addition, cardiac fibroblasts treated with Smad3 inhibitor also pointed to an important role of the lysyl oxidase (LOX) – an enzyme that is necessary for cross-linking of collagen proteins (Voloshenyuk *et al*., 2011). All of these data demonstrated the importance of the TGF-β-Smad axis in the development of cardiac fibrosis.

Smad-independent signalling

Stimulated TGF-β type I receptor activates not only the Smad-dependent response, but also triggers a number of Smad-independent signalling cascades, distinct from transcription. The signalling system involving phosphatidylinositol-3-kinase (PI3K) and protein kinase B, also known as Akt, represents one example of the TGF-β-induced signal transduction pathway, which is independent of Smad proteins. In this pathway, activated PI3K phosphorylates and activates the Akt kinase, which in turn modulates several downstream effectors, such as glycogen synthase kinase-3β (GSK-3β), mammalian target of rapamycin (mTOR) and many others (Oudit *et al*., 2004; Zhang, 2009; Peterson & Schreiber, 1998). The importance of PI3K/Akt signalling pathway has been implicated in specific aspects of cardiac fibrosis (Oudit *et al*., 2004). In particular, PI3K/Akt pathway was required for increased collagen synthesis in cardiac fibroblasts (Voloshenyuk *et al*., 2011). Furthermore, blockage of TGF-β dependent phosphorylation of Akt resulted in decreased expression of the LOX enzyme (Voloshenyuk *et al*., 2011). Inhibition of PI3K and Akt also indicated the importance of this pathway in TGF-β-dependent endoglin expression (Shyu *et al*., 2010). The PI3K/Akt pathway in cardiac fibrosis is, however, not regulated exclusively by TGF-β. The direct impact of this pathway on cardiac fibrosis was also proved upon interleukin (IL)-18 and β-adrenergic stimulation (Oudit *et al*., 2003; Fix *et al*., 2011).

Another example of Smad-independent TGF-β signal transduction is a signalling pathway dependent on the RhoA-ROCK axis (Heasman & Ridley, 2008). RhoA belongs to the Rho GTP-ase family and functions as a "switch-protein", transforming between active GTP-bound (RhoA-GTP) and inactive GDP-bound (RhoA-GDP) forms (Hubchak *et al*., 2009; Tsou *et al*., 2014; Zhan & Kanwar, 2014). The main effectors of RhoA pathway are Rho-associated, coiled-coil-containing protein kinases (ROCKs) existing in two isoforms, ROCK1 and ROCK2. ROCKs regulate various important cellular functions, including proliferation, migration, differentiation, cytoskeleton reorganisation and apoptosis (Shimizu & Liao, 2016). Experiments on various animal models indeed confirmed the importance of ROCKs in cardiac fibrogenesis. In ischemia-reperfusion model, ROCK1$^{-/-}$ mice showed a markedly reduced collagen deposition. Furthermore, ROCK1 was shown to mediate transformation of bone marrow precursors into fibroblasts (Haudek *et al*., 2009). ROCK1$^{-/-}$ mice also developed less perivascular and interstitial fibrosis in the pressure overload model induced by transverse aortic banding (Zhang *et al*., 2006). Experiments with haploinsufficient ROCK1$^{+/-}$ mice showed that partial reduction in ROCK1 levels was sufficient to prevent perivascular fibrosis induced by angiotensin II infusion, pressure overload or myocardial infarction (Rikitake *et al*., 2005). The profibrotic role of ROCK1 was also confirmed in mice overexpressing Gαq, which develop cardiomyopathy at an old age. In this model, deletion of ROCK1 reduced myocardial fibrosis, while cardiac-specific overexpression of ROCK1 caused acceleration of heart failure, cardiomyocyte apoptosis and fibrotic changes (Shi *et al*., 2010). In these fibrotic processes, ROCK1 is involved in myofibroblast differentiation and stress fibre formation (Hubchak *et al*., 2009; Shimizu & Liao, 2016). On the molecular level, ROCKs control polymerisation of monomeric G-actin into F-actin – a major component of stress fibres. ROCKs were also shown to release myocardin-related transcription factors (MRTF), which together with serum response factor (SRF) induce expression of the profibrotic genes (Small *et al*., 2010; Tsou *et al*., 2014).

Stimulation with TGF-β can also result in direct activation of non-canonical response dependent on mitogen-activated protein kinases (MAPKs), which transmit the signal from the cell membrane to the nucleus and regulate gene expression. Conventional MAPKs include the extracellular signal-regulated kinase 1 and 2 (Erk1/2 or p44/42), the c-Jun N-terminal kinases (JNKs) and the p38 isoforms (α, β, γ, and δ). Signal transduction typically consists of a core module of three sequentially phosphorylated kinases: MAPK, MAPK kinase (MAPKK), and MAPKK kinase (MAPKKK) (Petrich & Wang, 2004). MAPKs play important role in the effector response, as they regulate the activity of transcriptional cofactors cooperating with Smad proteins (Feng & Derynck, 2005; Lee *et al*., 2007). The functional contribution of MAPKs to cardiac fibrosis has been documented in the literature. In a mouse model of myocardial infarction, enhanced phosphorylation of Erk1/2 and JNK were reported to correlate with an increased degree of fibrosis (Sun *et al*., 2015), however other report confirmed an increased phosphorylation of p38 and Erk1/2, but not JNK (Yeh *et al*., 2010). In a rat model of pressure overload, all three major effector MAPKs: p38, Erk1/2 and JNK, became activated. Treatment with retinoic acid inhibited phosphorylation of these MAPKs and attenuated pathogenic cardiac remodelling (Choudhary *et al*., 2008). Recent data showed that inducible deletion of *Mapk14* (gene encoding p38α) in cardiac fibroblasts reduced the fibrotic response in post-infarcted heart (Molkentin *et al*., 2017). Study with TGF-β antagonist in spontaneously hypertensive rats confirmed involvement of p38 in cardiac fibrogenesis (Yan *et al*., 2009). In hypertensive heart, p38 is controlled by the injury responsive activating transcription factor 3 (ATF3) (Li *et al*., 2017). Furthermore, TGF-β signalling through p38 controls the transient receptor potential canonical 6 (TRPC6), which promotes conversion of cardiac fibroblast to myofibroblast (Davis *et al*., 2012). Furthermore, all three MAPKs: p38, Erk1/2 and JNK have been also suggested to regulate TGF-β-dependent LOX expression in cardiac fibroblasts (Voloshenyuk *et al*., 2011). Activation of Erk1/2-dependent pathway has been also functionally implicated in cardiac fibrosis. Mice with a laminin A/C gene mutation develop myocardial fibrosis, which depends on activation of the TGF-β-Erk1/2 axis. Interestingly, Erk1/2 activation was in part modulated by the TGF-β/Smad signalling (Chatzifrangkeskou *et al*., 2016). Moreover, TGF-β and Erk1/2 are also activated by high glucose in a mouse model of diabetic cardiomyopathy. Treatment with antioxidants suppressed activation of TGF-β and Erk1/2 and prevented development of myocardial fibro-

sis in this model (Wu *et al*., 2016). Recently, IL-11 was identified as a new player in cardiac fibrosis. IL-11 was essential for activation of non-canonical Erk pathway in TGF-β-mediated fibrosis (Schafer *et al*., 2017). In the signal transduction cascade, MAPKKK plays a central role in regulation of the MAPK activity. TGF-β-activated kinase 1 (TAK1) represents an important MAPKKK in TGF-β signalling (Biesemann *et al*., 2015). The relevance of TAK1-p38 axis activation in cardiac fibrogenesis was reported in myocardial infarction and pressure overload models (Matsumoto-Ida *et al*., 2006; Li *et al*., 2016). All of these data emphasize complexity of the Smad-independent response in the TGF-β-mediated cardiac fibrogenesis.

WNT SIGNALLING PATHWAYS IN CARDIAC FIBROSIS

WNT signalling represents another important signal transduction pathway which regulates organogenesis, cancer development and tissue fibrogenesis. The family of WNT ligands consists of highly evolutionarily conserved glycoproteins encoded in humans by 19 genes (Garriock *et al*., 2007). Upon translation, the WNT proteins undergo a series of post-translational modifications followed by a highly organized and tightly controlled exocytosis (Herr *et al*., 2012). Extracellular WNTs bind to transmembrane receptor complexes consisting of the Frizzled and the low-density lipoprotein receptor-related protein (LRP) families (Moon *et al*., 2004). After binding to receptor complexes, WNTs trigger various outputs in a β-catenin-dependent (canonical response) or independent (non-canonical response) manner. In the canonical response, binding of WNT1, WNT2b, WNT3a, WNT6 or WNT9b to the receptor complex activates the Dishevelled protein, which in turn inhibits GSK-3β-dependent degradation complex destroying the continuously synthetized β-catenin. This leads to stabilisation and translocation of β-catenin into the nucleus, where β-catenin acts as a transcriptional coactivator which together with the T-cell factor (TCF) and the lymphoid enhancer factor (LEF) transcription factors initiates transcription of the WNT target genes (Moon *et al*., 2004; Tao *et al*., 2016). Frizzled receptor stimulation with WNT4, WNT5a or WNT11 can trigger gene transcription independently of β-catenin by activating a planar cell polarity pathway and a calcium-dependent pathway. Signal transduction through the WNT/planar cell polarity pathway involves activation of certain MAPKs (JNK and ERK1/2 kinases) and the RhoA-ROCK axis. In the WNT/calcium-dependent pathway, signal transduction is instead mediated through phospholipase C (PLC) activation, followed by Ca^{2+} release. The increase in free cytoplasmic Ca^{2+} levels subsequently activates protein kinase C (PKC), calmodulin kinase II and a Ca^{2+}-sensitive enzyme, calcineurin. This might result in increased nuclear levels of transcription factor AP-1/c-Jun which regulates gene transcription (Bergmann 2010; Rao *et al*., 2010).

Natural regulation of the WNT pathway predominantly occurs at the level of extracellularly secreted inhibitors. One of the most important natural inhibitors of the WNT signalling belongs to the family of secreted Frizzled-related proteins (sFRPs), which directly bind to the WNT proteins and prevent their interactions with the Frizzled receptors or directly bind to the Frizzled receptors themselves (Cruciat & Niehrs, 2013). The Dickkopf proteins represent another family of secreted proteins negatively regulating WNT signalling. Dickkopf proteins present in the extracellular space bind to LRP 5/6 co-receptors triggering their internalization. Similarly, WNT-inhibitory factor 1 (WIF1) inhibits the WNT proteins' interaction with the receptor by binding to them (Surmann-Schmitt *et al*. 2009). Recently described WNT protein degrading enzymes, Tiki1 and Notum, represent another class of natural WNT inhibitors. Transmembrane metalloproteinase Tiki1 cleaves amino-terminal region of WNT ligands and thereby reduces their receptor-binding ability (Zhang *et al*., 2016). Similarly, a secreted Notum enzyme specifically removes an acyl group from the WNT proteins which is needed for effective binding of WNTs to the Frizzled receptors (Kakugawa *et al*., 2015). A natural regulation of WNT signalling is, however, not limited to inhibitors. Secreted R-spondin, for example, acts as a WNT ligand agonist enhancing ligand-receptor clustering and inhibits Frizzled and LRP receptor turn-over (Jin & Yoon, 2012).

Active WNT signalling has been implicated in cardiac tissue remodelling. For example, enhanced WNT1 expression was reported in the epicardium following acute ischaemic cardiac injury in mice (Duan *et al*., 2012), and increased levels of canonical (WNT2b, WNT9a) and non-canonical (WNT5a) WNTs were found in the myocardium of neonatal mice in response to cryoinjury (Mizutani *et al*., 2015). Increased activation of β-catenin and TCF/LEF have also been observed in human hearts with severe epicardial fibrosis (Ye *et al*., 2013). In recent years, a number of reports highlighted functional contribution of WNT signalling to the cardiac fibrogenesis in various animal models. In rodent models of myocardial infarction, elimination of bioavailable WNTs with sFRP1, sFRP2 or sFRP4 was shown to reduce fibrosis and improve cardiac function (Barandon *et al*., 2011; Fan *et al*., 2018; He *et al*., 2010; Matsushima *et al*., 2010). Blockade of the Frizzled receptors with a specific pharmacological antagonist also improved post-infarction cardiac function and reduced collagen content (Laeremans *et al*., 2011). Furthermore, in a mouse model of experimental autoimmune myocarditis, development of postinflammatory fibrosis was successfully prevented by administrating sFRP2 (Blyszczuk *et al*., 2017). Cardiac tissue remodelling is also regulated by other secreted natural negative regulators of WNT signalling. Dickkopf-3 has been shown to attenuate cardiac fibrosis in cardiac hypertrophy induced by pressure overload or infusion with angiotensin II (Zhai *et al*., 2018; Zhang *et al*., 2014).

Regulation of WNT signalling in cardiac fibrosis is mediated not only through secreted factors, but also intracellularly. Constitutive overexpression of the Dishevelled protein (which inhibits GSK-3β) activated canonical and non-canonical WNT signalling pathways and thereby induced spontaneous myocardial fibrosis and cardiac hypertrophy (Malekar *et al*., 2010). Recent data specifically pointed to the important role of the canonical WNT signalling in cardiac fibroblasts. In myocardial fibrosis induced by transaortic constriction, genetic depletion of β-catenin in cardiac fibroblasts reduced interstitial fibrosis, although did not alter the number of activated cardiac fibroblasts (Xiang *et al*., 2017). In the complementary approach, Lal and coworkers (Lal *et al*., 2014) demonstrated that activation of the canonical WNT pathway by inhibiting GSK-3β in cardiac fibroblasts promoted fibrogenesis in postinfarcted hearts.

In the context of myocardial fibrosis, at the cellular level, the WNT proteins have been mainly implicated in the activation of cardiac fibroblasts. WNT1 was shown to stimulate cardiac fibroblast proliferation and to upregulate profibrotic genes, including collagen I and endothelin-1 (Duan *et al*., 2012). Such profibrotic response

in cardiac fibroblasts was also induced by WNT1-induced secreted protein-1 (WISP-1) (Colston *et al.*, 2007). Combined co-transfection of Frizzled receptors and stimulation with WNT3a or WNT5a indicated the involvement of the canonical and non-canonical WNT pathways during cardiac fibroblast differentiation (Laeremans *et al.*, 2010). Stimulation of cardiac fibroblasts with WNT5a showed Erk-dependent production of IL-6 and tissue inhibitor of metalloproteinase 1 (TIMP-1) (Abraityte *et al.*, 2017). Other data showed that stimulation by WNT1/WNT5a or WNT3a ligands alone were insufficient to induce effective pathological myofibroblast formation (Blyszczuk *et al.*, 2017). Similarly, overexpression of β-catenin had no effect on myofibroblast lineage differentiation (Laeremans *et al.*, 2010), but induced proliferation and suppressed apoptosis of the cardiac fibroblasts (Hahn *et al.*, 2006).

Bioactivity, as well as transcriptional regulation of WNTs, are tightly controlled by sFRPs representing an important arm of the WNT system regulation (Cruciat & Niehrs, 2013; Sklepkiewicz *et al.*, 2015). Accordingly, dysregulation of WNT-sFRP balance can affect fibrotic processes in the heart. Mice lacking sFRP1 showed increased expression of WNT ligands, elevated levels of β-catenin, and enhanced αSMA expression and collagen production in cardiac fibroblasts (Sklepkiewicz *et al.*, 2015). Although elimination of bioavailable WNTs with exogenous sFRP2 reduced cardiac fibrosis *in vivo* (Blyszczuk *et al.*, 2017; He *et al.*, 2010), constitutive expression of sFRP2 in cardiac fibroblasts surprisingly activated the WNT/β-catenin signalling and promoted fibroblast proliferation and expression of the ECM genes (Lin *et al.*, 2016). Furthermore, sFRP2-deficient cardiac fibroblasts produced less collagen and sFRP2-deficient mice developed less fibrosis following myocardial infarction (Kobayashi *et al.*, 2009). These data suggest that constitutive role of sFRP2 in cardiac fibroblasts is far more complex than suppression of profibrotic WNT signalling.

TGF-β-WNT CROSSTALK IN FIBROGENESIS

As demonstrated above, activation of the TGF-β and WNT signalling pathways plays important roles in fibrotic processes in the heart. So far, our understanding of the direct interplay between these two pathways, specifically in the cardiac fibrogenesis, is limited.

Regulation of TGF-β by WNT

Little is known about regulation of the TGF-β production by WNTs in the fibrotic process. Experiments on cardiac fibroblasts suggested that neither canonical nor non-canonical WNT signalling directly regulated TGF-β at the mRNA level (Laeremans *et al.*, 2010). Canonical WNTs also failed to upregulate TGF-β in the lung fibroblasts (Lam *et al.*, 2011). However, β-catenin-dependent WNT3a upregulated TGF-β production in mouse embryonic fibroblasts (Carthy *et al.*, 2011). Interestingly, in the cardiac fibroblasts, β-catenin signalling pathway was reported to control TGF-β production induced by fibroblast growth factor 23 (Hao *et al.*, 2016). Furthermore, insight from non-cardiac models demonstrated transcriptional regulation of PAI-1 by WNT1 in kidney epithelial cells, suggesting a potential negative regulation of TGF-β signalling by canonical WNT (He *et al.*, 2010).

Regulation of canonical WNT by TGF-β

Regulation of the WNT/β-catenin pathway by TGF-β is much better documented. TGF-β has been implicated in production and secretion of the WNT proteins through a TAK1-dependent pathway in cardiac fibroblasts and in heart inflammatory cells (Blyszczuk *et al.*, 2017). Accordingly, in a mouse model of experimental autoimmune myocarditis and in a mouse model of pressure overload, impaired profibrotic TGF-β responses were observed in the absence of β-catenin or upon pharmacological inhibition of the β-catenin-dependent signalling, pointing to the involvement of canonical WNT pathway in this process (Blyszczuk *et al.*, 2017; Xiang *et al.*, 2017). Furthermore, TGF-β inducible PI3K/Akt pathway was also reported to inhibit GSK-3β (enzyme involved in β-catenin degradation) activity in the cardiac fibroblasts (Ma *et al.*, 2017). Thus, TGF-β might directly activate the β-catenin-dependent pathway.

It has been shown that canonical WNT signalling can control profibrotic TGF-β response. Cardiac fibroblasts lacking GSK-3β showed enhanced profibrotic response and activation of Smad3 signalling, leading to enhanced cardiac tissue remodelling in a mouse model of myocardial infarction (Lal *et al.*, 2014). In addition, sirtuin 3 has been proposed to suppress profibrotic TGF-β signalling through activation of GSK-3β in cardiac fibrosis (Sundaresan *et al.*, 2016). These results suggested that β-catenin-dependent signalling also contributed to stabilization of the canonical TGF-β response. Activation of the canonical WNT signalling in response to TGF-β and the functional contribution of this mechanism to fibrotic processes have been observed not only during cardiac fibrogenesis, but also in other organs, including skin (Akhmetshina *et al.*, 2012), lungs (Xu *et al.*, 2017) and the hair follicles (Lu *et al.*, 2016; Si *et al.*, 2017, Plikus *et al.*, 2012). Summarising, these data pointed to the important role of β-catenin-dependent pathway in TGF-β response. In the proposed mechanism, TGF-β activates WNT/β-catenin signalling through production of WNT proteins and by direct deactivation of GSK-3β. Activated WNT/β-catenin, in turn, stabilizes the TGF-β/Smad response. It seems that co-activation of these two pathways is required to trigger the effective fibrotic response.

Regulation of non-canonical WNT by TGF-β

In contrast to quite well-documented involvement of the canonical WNT signalling in the TGF-β response, much less is known about contribution of the non-canonical WNT pathway. Although elevated levels of non-canonical WNT5a were detected in fibrotic hearts in mice and in humans (Abraityte *et al.*, 2017; Blyszczuk *et al.*, 2017), the interplay between TGF-β and WNT5a (or other non-canonical WNTs) remains mainly speculative. Noteworthy, stimulation of the planar cell polarity pathway by non-canonical WNTs is known to activate MAPKs and Rho-dependent ROCKs, and thereby to regulate cytoskeletal organization and fibrotic response (Bergmann, 2010; Abraityte *et al.*, 2017). As presented above, TGF-β also activates MAPK and ROCK pathways in a Smad-independent manner. It is possible that in the TGF-β stimulated cells, non-canonical WNTs stabilize or modulate p38-, Erk-, JNK- and ROCK-dependent responses in the fibrotic processes. However, the ultimate effect of such co-activation needs to be experimentally addressed in the future.

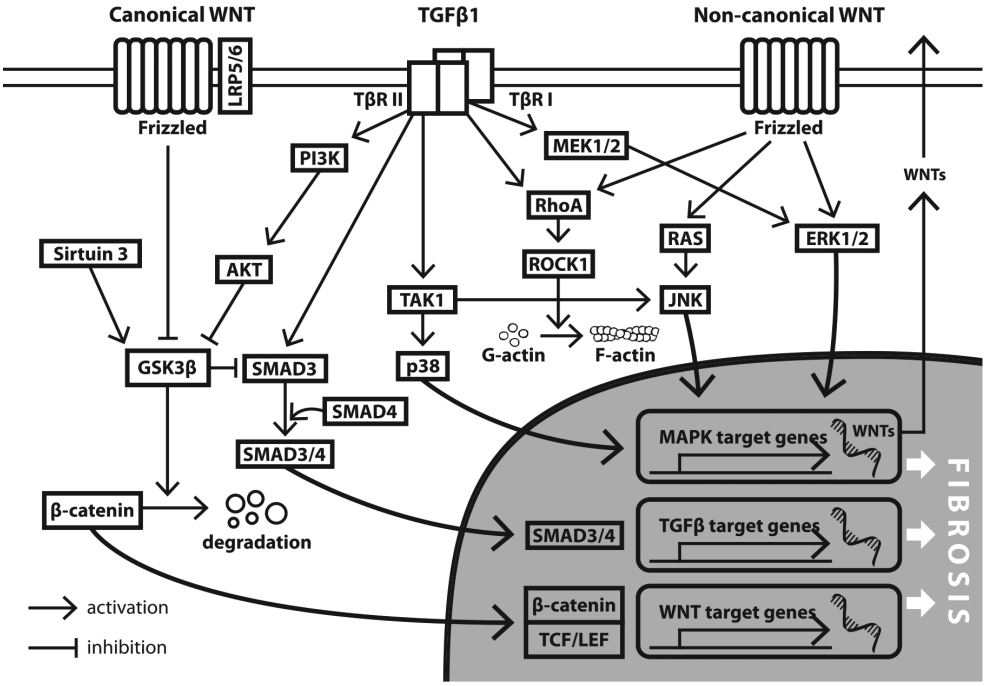

Figure 1. Schematic representation of the proposed crosstalk between TGF-β and WNT signalling pathways in cardiac fibrogenesis. TGF-β activates WNT/β-catenin signalling through production and secretion of WNTs, and by direct deactivation of GSK-3β. Activated WNT/β-catenin in turn stabilizes TGF-β/Smad response. In addition, non-canonical TGF-β (Smad-independent) together with non-canonical WNT (β-catenin-independent) activates MAPKs (p38, JNK and Erk1/2) and RhoA-ROCK. These mechanisms induce expression of profibrotic genes which initiate fibrotic changes at the cellular and tissue levels.

CONCLUSIVE REMARKS

Progressive fibrosis is a driving pathological force in many cardiac conditions. Understanding molecular mechanisms of this process could provide solutions to prevent progression or even to revert fibrotic changes in the heart. Mechanisms of cardiac fibrogenesis are rather complex and involve multiple players. In the light of the large number of identified profibrotic factors, understanding of interplays between these signalling pathways represents an emerging challenge in this field. As presented here, accumulating evidence points to the critical role of TGF-β and WNT pathways in the pathogenesis of fibrosis in the heart and in other organs. Although biosynthesis of TGF-β and WNTs is quite well described, the bioavailability of these profibrotic factors in various cardiac pathologies remains largely elusive. Unlike typical cytokines and chemokines, biological activity of TGF-β and WNTs is strongly regulated in the extracellular space. Future research should focus more on identifying mechanisms regulating bioavailability of these profibrotic agents.

As discussed in this review, the crosstalk between TGF-β and WNT pathways seems to be essential in switching on the genetic machinery of the profibrotic changes. The crosstalk between these two pathways includes not only positive feedback loops, but also common downstream signalling molecules and outputs (Fig. 1). Experimental data demonstrated that only highly organised and coordinated response of these multifaceted mechanisms effectively translates into fibrotic changes at the cellular and tissue levels. It is important to note that both, the TGF-β and WNT pathways, regulate a number of various cellular processes and play fundamental roles in organogenesis and in carcinogenesis. Thus, consequences of uncontrolled systemic dysregulation of these signalling pathways might be fatal. It seems that precise targeting of specific aspects of the TGF-β-WNT signalling network, rather than its global inhibition, is a better strategy for the development of successful side effect-free antifibrotic approaches. Identification of these new targets requires, however, a better understanding of molecular mechanisms.

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Disclosures

None

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